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THE IMPLICATION OF TRYPTOPHAN PYRROLASE IN ENDOTOXIN POISONING

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(Prepared under Contract AF 41 (609)-1764 by
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SECTION 1. INTRODUCTION

The ability of certain adrenocortical hormones to protect experimental animals against the lethal effects of lipopolysaccharides derived from Gram negative bacteria is well established (Duffy and Morgan, 1951; Geller et al, 1954; Thomas and Smith, 1954; Levitin et al, 1956). The underlying basis for this protection has not been determined. Brooke et al (1961) recently reported for several adrenocorticoids a lack of correlation between their anti-inflammatory properties and their ability to protect against endotoxins. It is perhaps a matter of faith that leads one to the view that all such hormonal capabilities (and of other substances with pharmacological actions as well) are ascribable to primary biochemical or metabolic effects. The problem becomes a matter of detecting the significant enzymic "needle" from among the mammalian "haystack" of enzymes. A number of enzymes may become altered by hormonal injections after a sufficient delay but most of these changes are probably secondary or adaptive rather than primary. A hopeful way out of this challenging situation is to find alternate ways of modifying an enzyme to see if by both methods a protective effect against endotoxins results.

Such an approach to the problem of corticoid protection against endotoxin is made possible by the work of Knox and Auerbach (1955) and Feigelson et al (1962). These investigators have shown that an injection of cortisone into rats is followed within about four hours by a significant increase in liver tryptophan pyrrolase. The enzyme also becomes more active following an injection of tryptophan, probably as a result of induced enzyme formation.

Very briefly, tryptophan pyrrolase, with hematin as cofactor, oxidatively converts tryptophan into kynurenine which, in turn, is further degradated into nicotinic acid. The ultimate metabolic fate of tryptophan via this pathway is the incorporation of nicotinamide into the di- and triphosphopyridine nucleotides (DPN and TPN), compounds of major significance in the energy release by organisms.

If an elevation in activity of tryptophan pyrrolase results in metabolic reactions of survival value in endotoxin poisoning, then tryptophan alone should protect mice against endotoxin to a degree somewhat commensurate with the protection afforded by any one of several adrenocorticoids. Moreover, the products yielded by the enzyme should also act similarly to the

corticoids and to tryptophan if the reactions are of importance in an animal's response to endotoxin. With these considerations serving to guide the research, the results to be presented in this report were obtained.

SECTION 2. METHODS

Endotoxins. As in previous reports (Berry and Smythe, 1959, 1961), the standard material employed as endotoxin is a saline suspension of heat-killed (pasteurized) Salmonella typhimurium, strain SR-11. A sufficient quantity of material for several months of work was prepared, dispensed in sealed tubes in 10 ml portions and stored at 5° C until ready for use. The dry weight of the suspension was 6.0 mg per ml and the number of bacteria, as estimated by a dilution count prior to heating, was approximately 8×10^9 per ml. A soluble purified lipopolysaccharide (LPS) derived from Escherichia coli (02:K1, lot No. 3712:92a, generously supplied by Dr. C. W. deWitt, Upjohn Co., Kalamazoo) was used for comparative purposes. In some experiments LPS derived from Serratia marcescens (generously supplied by Dr. A. Nowotny, Temple University) was employed.

Protection Experiments. Two types of protection experiments were carried out. In the first, the test material and the endotoxin were each injected intraperitoneally (cortisone was given subcutaneously) within a time interval no greater than 10 to 15 minutes. In the second series of experiments, the endotoxin was given four hours before the test material, which was sometimes given again eight hours after the endotoxin. In this situation the aim was to improve the condition of an animal already showing symptoms of endointoxication, whereas with concurrent injections the objective was to prevent the onset of illness.

All injections were given in a volume of 0.5 ml and all solutions were made up in pyrogen free saline (Baxter Laboratories, Morton Grove, Ill.). Cortisone acetate (Nutritional Biochemicals Corp., Cleveland) was given in 5 mg amounts. Nicotinamide, 10 mg, nicotinic acid, 10 mg, and l-tryptophan, 20 mg (all from Nutritional Biochemical Corp., Cleveland) were also injected. Diphosphopyridine nucleotide (DPN) (Sigma, St. Louis) was given in 5 mg and 10 mg amounts. In some experiments, glucose was combined with one of the above reagents in 45 mg quantities and in one series it was added to 5 mg of adenosine triphosphate (ATP) (Sigma, St. Louis). The survival data are based on an observation period of 48 hours.

Tryptophan Pyrrolase Assay. The method used by Knox and Auerbach (1955) for the assay of tryptophan pyrrolase in rat liver was modified for

ABSTRACT

An attempt has been made to relate the protective effect of cortisone against lethality of bacterial endotoxins in mice to changes in activity of liver tryptophan pyrrolase (TP), an enzyme known to be activated by the hormone. In mice maintained at 25° C, TP activity remained normal under conditions associated with survival and diminished in activity when animals were not protected. In addition, nicotinamide and diphosphopyridine nucleotide (DPN) were found to protect mice at 25° C against lethality of endotoxin about as effectively as cortisone. At 5° C, however, mice could not be protected against death from endotoxin by DPN and even cortisone and nicotinamide were less effective than at 25° C. Mice in the cold die sooner than and with different symptoms from those at room temperature.

PUBLICATION REVIEW

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Director of Research

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Tryptophan Pyrrolase Assay. The method used by Knox and Auerbach (1955) for the assay of tryptophan pyrrolase in rat liver was modified for

application to mice. Livers were removed and homogenized immediately with a Teflon and Pyrex homogenizer in cold KCl (0.14 M) made alkaline with NaOH (0.0025 N). Homogenates were kept in an ice-water bath until time of addition of an aliquot to the incubation flasks.

Reaction vessels (25 ml Erlenmeyer flasks equipped with stoppers for gassing) containing reagents were permitted to equilibrate at 38° C in a water bath with reciprocating shaker (New Brunswick Instrument Co.) during the time required for the homogenization of livers. The flasks contained phosphate buffer, 0.2 M and pH 7.0, L-tryptophan, 0.03 M, and distilled water to a final volume of 8.0 ml. The liver homogenate to be assayed was added last and the flasks were gassed for five minutes with 100% oxygen. At the end of one hour in the shaker, the reaction was stopped by the addition of metaphosphoric acid. A "blank" determination was carried through for livers in each experimental group by omitting the tryptophan substrate from a flask. Spectrophotometric readings for these determinations were constant (within less than 2% variation) except under experimental conditions where the tryptophan pyrrolase activity was extremely high. Under these conditions the "blank" was increased to about 10% of the control values. In all cases, flasks with substrate were read against those to which no substrate was added.

Known amounts of kynurenine sulfate (0.5 - 5.0 μM per flask) were equilibrated in the presence of liver homogenate from normal animals and carried through the entire procedure. All reaction filtrates were neutralized with NaOH to pH 6.5 - 7.5 and read in a Beckman DU spectrophotometer at 360 mμ. Dry weights were determined for each sample of liver homogenate and enzyme activity was expressed as μM kynurenine formed per gram dry weight of liver per hour.

Assay for Pyridine Nucleotides. A sample of liver, 300 to 500 mg, was removed, weighed and immediately homogenized in 5 ml of 5% trichloroacetic acid in a glass tube with Teflon pestle. The precipitated proteins were removed by centrifugation at 2200 X g for 20 minutes and aliquots of the clear supernatant were used for fluorescence measurements.

The method of Kaplan et al (1951) was followed for the quantitative determination of the oxidized pyridine nucleotides. These substances form stable fluorescent compounds in the presence of strong (5N) alkali. DPN (Sigma) was used for the standard curve in the range of 0 to 1 μg/ml. All solutions were heated in a boiling water bath for five minutes to develop maximum fluorescence. A Turner fluorometer with a primary filter, number 7-60 (360 mμ exciting light), and a secondary filter, number 75 (490 mμ transmitted light), was used to measure fluorescence.

This method does not detect the reduced pyridine nucleotides (DPNH and TPNH) since they are rapidly inactivated during the acid extraction of the tissues (Lowry et al, 1957). However, nicotinamide mononucleotide and nicotinamide riboside, like DPN, form fluorescent products on treatment with strong alkali. Methyl nicotinamide also contributes some fluorescence but only about 1/60 that of DPN.

Exposure of Mice to Cold (5° C). Mice were placed in individual plexiglass compartments free of bedding and with the floor of the cage made of one-quarter inch hardware cloth so that no moisture accumulated. Each mouse was provided with food and water and the cages were placed on the shelves of a walk-in refrigerator maintained at 5° ± 1° C. The animals were protected from drafts. Twelve hours of light and 12 hours of dark were assured by an automatic switch. No control over relative humidity was attempted.

Mice. CF-1 female mice (Carworth Farms, New City, New York) were received weekly when weighing 16 to 18 g. They were housed 10 per cage with pine shavings as bedding and were fed Dietrick and Gambrill's (Frederick, Md.) pathogen-free mouse food. After one to two weeks, when they weighed 20 ± 1 g, they were subjected to the different experimental procedures. The animal room and the laboratory in which they were held during experimental observation were both maintained at 25° ± 2° C. Food and water were available at all times unless specifically stated.

SECTION 3. RESULTS

Protection Experiments with Concurrent Injections of Endotoxin and Metabolites

In Table I a comparison is made of the ability of different substances to protect mice against graded amounts of endotoxin administered in the form of heat-killed S. typhimurium. The dosages of endotoxin administered to control mice range from, approximately, 20% lethal to 100% lethal. These animals were also injected with 0.5 ml saline since treated animals received this volume of fluid containing the protective agent. Results with cortisone, the one substance known for more than a decade to be highly protective against the lethal and other manifestations of endotoxin poisoning, are shown in the second line of the table. Nicotinamide, at a molar quantity four times that of cortisone, affords almost the same degree of protection as the hormone. Nicotinamide is of interest because it is part of the molecule of the pyridine nucleotides and is synthesized along the metabolic

pathway initiated by tryptophan pyrrolase. Interestingly enough, however, nicotinic acid, which is formed prior to the amide, not only fails to protect against endotoxin but potentiates it. Thus, the dose that is only 20% lethal in control mice is 100% lethal in mice given nicotinic acid. Possibly the vasodilator effect of the acid is involved or it may be the result of a specific metabolic effect, as mentioned in a later section of the report.

TABLE I

Protection of mice against heat-killed S. typhimurium with compounds related to tryptophan pyrrolase activity. All injections were given intraperitoneally except cortisone, which was given subcutaneously. The endotoxin and the protective agent were injected in rapid succession. The P-values, compared with controls, were calculated by the rank order test of Wilcoxon (1949).

Substance Injected at Time of Endotoxin	Number of Mice Surviving/Total Injected with Weight of Heat-Killed S. typhimurium Shown			
	0.75 mg	1.5 mg	3.0 mg	4.5 mg
Control, 0.5 ml Saline	8/10	15/30	2/30	0/20
Cortisone, 5 mg (19 μM)		20/20 P=.008	15/20 P<.008	9/20 P=.014
Nicotinamide, 10 mg (82 μM)	10/10 N.S.*	20/20 P=.008	13/20 P<.008	5/20 P>.05
Nicotinic Acid, 10 mg (81 μM)	0/10 P<.008	0/10 P<.008		
DPN, 10 mg (15 μM)		20/20 P=.008	14/20 P<.008	5/20 P>.05
DPN, 5 mg (7.5 μM)		18/20 P=.028	8/20 P=.057	

* N.S. = Not statistically significant.

DPN at two dosage levels, about the same or slightly less in micromolar amounts than that of cortisone, affords good protection against endotoxin. This finding is consistent with the postulate that cortisone acts through its ability to increase the activity of tryptophan pyrrolase which, in turn, results in greater tissue levels of DPN and other nucleotides. (This suggestion is confirmed by data presented in Table VIII.)

When purified lipopolysaccharide derived from E. coli was substituted for heat-killed S. typhimurium as a source of endotoxin, the protective effect of the same compounds was observed. The results are summarized in Table II and show that both nicotinamide and DPN are equally active while nicotinic acid potentiates the lethal properties of the lipopolysaccharide. Tryptophan was without significant effect.

TABLE II

Protection of mice against E. coli lipopolysaccharide with compounds related to tryptophan pyrrolase activity. All injections were given intraperitoneally except cortisone, which was given subcutaneously. The endotoxin and the protective agent were injected in rapid succession. The P-values, compared with controls, were calculated by the rank order test of Wilcoxon (1949).

Substance Injected at Time of Endotoxin	Number of Mice Surviving/Number Injected with 175 μg E. coli Lipopolysaccharide
Control, saline 0.5 ml	9/20
Nicotinamide, 10 mg (82 μM)	17/20 P = 0.028
Nicotinic Acid, 10 mg (81 μM)	2/20 P = 0.057
Tryptophan, 20 mg (100 μM)	14/20 N.S.*
DPN, 10 mg (15.1 μM)	17/20 P = 0.028

* N.S. = Not statistically significant.

The inability of tryptophan to protect mice against endotoxin death when given either concurrently or four hours before heat-killed S. typhimurium is shown clearly in the data of Table III. These findings are contrary to expectations since tryptophan is known from the literature to inductively elevate pyrrolase activity in normal rats (Knox and Auerbach, 1955; Feigelson et al, 1962). As Figure 1 shows, this also occurs in unpoisoned mice. The increase in enzyme activity is linear with dosage of tryptophan over the range studied.

Of even greater interest is the obvious potentiation of endotoxin by tryptophan when the amino acid is given four hours after the heat-killed cells (last line of Table III). An LD_{50} dose in control mice becomes an LD_{100} dose following the delayed injection of tryptophan. A possible explanation for this action of tryptophan is given below.

In order to avoid the possibility that the results described above were unique for CF-1 mice, duplicate experiments were carried out with animals (Swiss-Webster mice) purchased from another dealer (Dierolf Farms, Boyertown, Pa.). These mice were protected against S. marcescens LPS with nicotinamide, DPN and cortisone to about the same degree as CF-1 animals. The lethal effect of endotoxin was potentiated by nicotinic acid and by tryptophan given four hours after the LPS. Thus, in respect to lethality, the Dierolf mice responded the same as CF-1 mice.

TABLE III

Treatment of mice injected with heat-killed S. typhimurium with tryptophan administered four hours before, at time of, and four hours after the endotoxin. All injections were given intraperitoneally. The P-values, compared with controls, were calculated by the rank order test of Wilcoxon (1949).

Times of Tryptophan and Endotoxin Injection	Number of Mice Surviving/Number Injected with 1.5 mg of Heat-Killed S. typhimurium
Control, saline 0.5 ml at time of endotoxin	15/30
Tryptophan, 20 mg Four hours before endotoxin	14/20 N. S. *
Tryptophan, 20 mg At time of endotoxin	11/30 N. S.
Tryptophan, 20 mg Four hours after endotoxin	0/20 P = 0.008

* N. S. Not statistically significant.

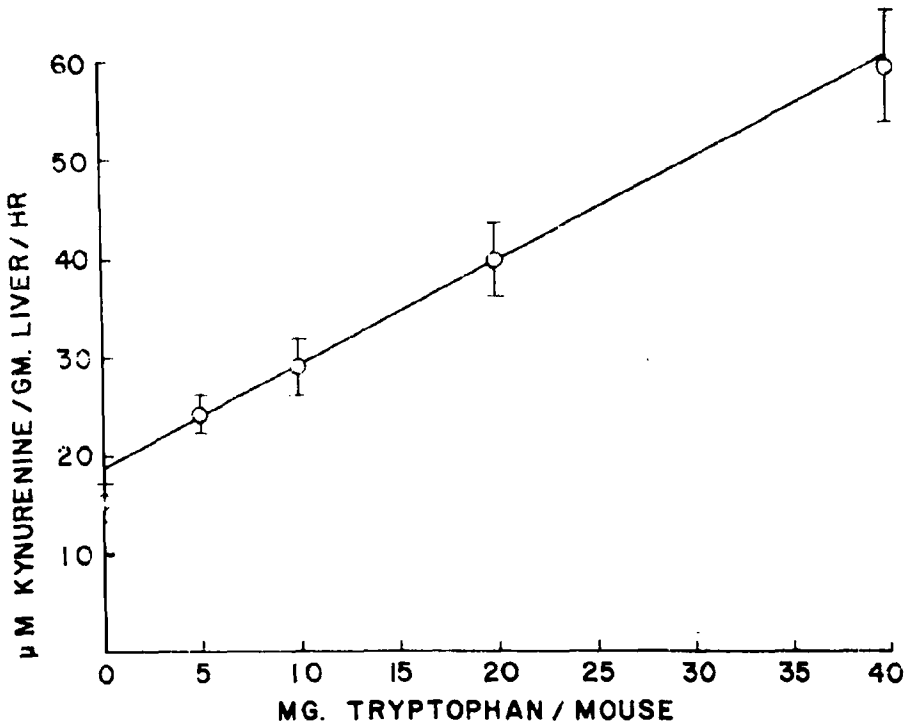

FIGURE 1

Liver tryptophan pyrrolase activity in mice four hours after intraperitoneal injection of amounts of tryptophan shown.

Irreversibility of Endointoxication

It has been found that cortisone (and other glycocorticoids) has prophylactic value against endotoxin but is unable to protect against lethality after a delay of a few hours (Berry and Smythe, 1961). Endointoxication appears to be irreversible, therefore, as far as cortisone is concerned. It was considered worthwhile, in light of the data of Table I, to examine the possible benefit that compounds formed beyond the metabolic conversion catalyzed by tryptophan pyrrolase might have after the animal begins to show symptoms of endotoxin poisoning. The results are presented in Table IV. None alone nor in combination with glucose was effective nor did any appear to potentiate endotoxin in a manner similar to tryptophan. Glucose was added to the metabolite since previous work from this laboratory (Berry et al, 1959) and elsewhere (Kun and Miller, 1948; Kun, 1948) has established the fact that endotoxin rapidly depletes carbohydrate reserves in the mouse and in other laboratory animals. The possibility exists, therefore, that available metabolic energy in the poisoned animal would be increased by the addition of substrate. No improvement in survival of the mice is evident. Cortisone, also, fails to protect (last line, Table IV) when given four hours after endotoxin.

Tryptophan Pyrrolase Activity in Livers of Endotoxin Poisoned Mice

Figure 2 presents in graphic form the tryptophan pyrrolase activity in livers of mice following a single injection of an LD_{50} dose of heat-killed S. typhimurium. An initial increase within four to six hours results in a significant rise in enzyme activity. At 17 hours, however, there is less than half the control level of pyrrolase action and even after 48 hours the enzyme has not returned to normal. This sequence of changes is made more understandable by the results presented below.

Adrenalectomized mice, maintained on 1% sodium chloride solution, were subjected to the water excretion test of Beatty et al (1954) as a measure of adrenal deprivement. Animals which were considered to be completely adrenalectomized on the basis of this test were then used for the determination of liver tryptophan pyrrolase activity one to two weeks post-adrenalectomy. One group of mice was kept as controls and another group was injected intraperitoneally with heat-killed S. typhimurium at an LD_{50} dose level for normal animals. This is approximately 250 times the LD_{50} for adrenalectomized mice. Four hours after the endotoxin was administered, the livers were assayed for tryptophan pyrrolase. The data of Table V summarize the findings. The enzyme activity in the adrenalectomized mice is found in control animals, but there is no change in the level of enzyme four hours after injection of endotoxin in contrast to the increase observed in the intact animal. Apparently, therefore, the sequence of changes seen in Figure 2 is dependent in part on adrenal response. This is further borne out by the results now to be described.

TABLE IV

Delayed treatment of mice injected with heat-killed S. typhimurium. All injections were given four hours and eight hours after the endotoxin (cortisone four hours only) and all were injected intraperitoneally (cortisone, subcutaneously). The P-values, compared with controls, were calculated by the rank order test of Wilcoxon (1949).

Substance(s) Injected Four and Eight Hours after Endotoxin	Number of Mice Surviving/Number Injected with Weight of Heat-Killed S. typhimurium Shown	
	1.5 mg	3.0 mg
Control, Saline 0.5 ml	19/30	4/30
Nicotinamide, 10 mg	13/20 N. S.*	5/20 N. S.
Nicotinamide, 10 mg + Glucose, 25 mg	10/20 N. S.	5/20 N. S.
DPN, 5 mg	10/20 N. S.	1/20 N. S.
DPN, 5 mg + Glucose, 25 mg	9/20 N. S.	0/20 N. S.
ATP, 5 mg + Glucose, 25 mg	18/20 N. S.	4/30 N. S.
Cortisone, 5 mg at four hours only	11/20 N. S.	4/20 N. S.

* N. S. Not statistically significant.

TABLE V

Effect of heat-killed S. typhimurium on tryptophan pyrrolase activity of liver from mice adrenalectomized one to two weeks previously. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental Treatment	Tryptophan Pyrrolase Activity of Liver (μM Kynurenine/g Liver/Hour)
Adrenalectomized mice	7.2 ± 0.8 (8)
Adrenalectomized mice four hours after 1.5 mg of heat-killed S. typhimurium	7.0 ± 1.0 (5)

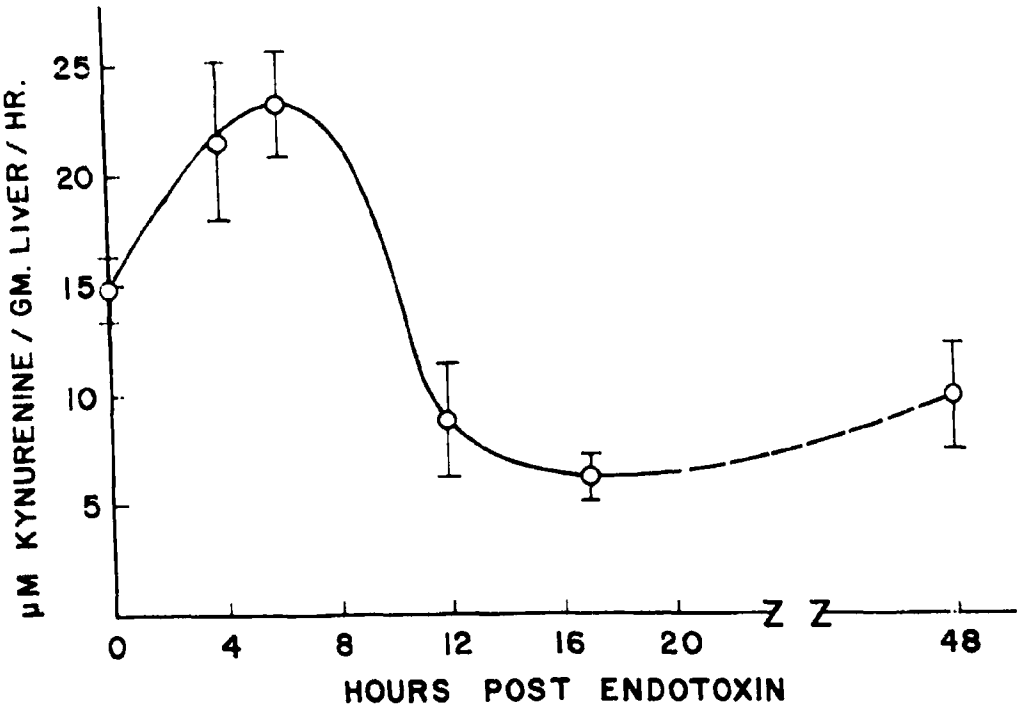

FIGURE 2. Liver tryptophan pyrrolase activity in mice at times indicated following intraperitoneal injection of an LD_{50} of heat-killed S. typhimurium.

Effect of Experimental Treatments on Tryptophan Pyrrolase Activity in Livers of Mice

The first two lines of Table VI give the numerical values plotted graphically in Figure 2 at time zero and 17 hours after an injection of an LD_{50} dose of endotoxin. The poisoned mouse at 17 hours shows typical symptoms of endointoxication: conjunctivitis, diarrhea, prostration, ruffled fur, weight loss, etc. At this time, tryptophan pyrrolase activity is less than one-half normal and is not significantly different from that of the adrenalectomized mouse (Table V). Animals injected with the same amount of endotoxin and concurrently with 5 mg of cortisone acetate are completely protected (Table I) and have normal tryptophan pyrrolase activity (line 3, Table VI). This is not observed, however, when 20 mg of tryptophan are substituted for cortisone. The mice are not protected (Table II) and, as line 4 of Table VI shows, neither is tryptophan pyrrolase activity. The fact that both cortisone and tryptophan result in similar increases in the enzyme when given singly to control animals is evident from the values at the bottom of Table VI. These were measured four hours postinjection. Neither substance maintains enzyme activity 17 hours after it is injected. One may presume, therefore, that the low tryptophan pyrrolase level 17 hours after endotoxin alone or in combination with tryptophan may be associated with adrenal insufficiency. Cortisone, by contrast, permits normal enzyme levels and, by inference, normal adrenal function to be maintained in endotoxin poisoned mice.

Tryptophan Pyrrolase Activity in Tolerant Mice

Mice were made tolerant to endotoxin by a series of injections of heat-killed S. typhimurium. The first two days they were given 0.1 LD_{50} (~.15 mg dry wt). This was followed by 0.2 LD_{50} on days three and four, and by 0.4 LD_{50} on days five and six. When challenged on days eight and 10 with E. coli lipopolysaccharide, the LD_{50} was increased five-fold. Assays for tryptophan pyrrolase were carried out on day eight on tolerant mice fasted for 17 hours and on another group 17 hours after an LD_{50} of S. typhimurium for nontolerant mice. The results are presented in Table VII and show no significant difference between the levels of enzyme activity. The control value for tolerant mice (12.4) is significantly less than that for normal mice (14.9, line 1, Table VI) at the 1% probability level as determined by rank order test (White, 1952). The enzyme had apparently failed to return completely to normal.

TABLE VI

Effect of heat-killed S. typhimurium, tryptophan and cortisone singly and in combination on tryptophan pyrrolase activity of liver from mice. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental Treatment	Tryptophan Pyrrolase Activity of Liver (μM Kynurenine/g Liver/Hour)
Control-fasted 17 hours	14.9 ± 1.5 (16)
17 hours after an LD_{50} (1.5 mg) dose of endotoxin	6.0 ± 1.0 (17)
17 hours after endotoxin + 5 mg cortisone	13.3 ± 1.7 (6)
17 hours after endotoxin + 20 mg tryptophan	6.2 ± 1.0 (6)
Four hours after 5 mg cortisone	43.5 ± 2.8 (7)
Four hours after 20 mg tryptophan	39.7 ± 4.1 (4)

TABLE VII

Effect of Heat-Killed S. typhimurium on tryptophan pyrrolase activity of liver from tolerant mice. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental Treatment	Tryptophan Pyrrolase Activity of Liver (μM Kynurenine/g Liver/Hour)
Tolerant mice - 17 hours fasting	12.4 ± 2.4 (9)
Tolerant mice - 17 hours after 1.5 mg heat-killed S. typhimurium	11.8 ± 1.8 (6)

Pyridine Nucleotide Levels in Liver

A change in tryptophan pyrrolase activity may be expected to result in a change in total pyridine nucleotides. This has been found to be true as the data of Table VIII show. Endotoxin poisoned mice at 17 hours have about two-thirds the amount of nucleotides as control animals. This is a larger fraction than was found for the enzyme (see Table VI) but is perhaps dependent, at least in part, on the timing of the changes since a decrease in the enzyme should precede that of the coenzymes. Both cortisone and nicotinamide result in a significant increase in pyridine nucleotides but the latter is twice as effective as the former. This may be related to the fact that nicotinamide is part of the nucleotide molecule. Kaplan et al (1956) have reported similar findings and have also shown that nicotinic acid has little stimulatory effect on the biosynthesis of pyridine nucleotides. It may be that this effect of nicotinic acid plays an important role in potentiating endotoxin (see above).

Concurrent injection of cortisone or nicotinamide with endotoxin protects mice against lethality (Table I), against a decrease in tryptophan pyrrolase activity (Table VI, cortisone only) and, as the last two lines of Table VIII show, against a change in total oxidized pyridine nucleotides. The necessity of a normal level of coenzymes for survival of an endotoxin poisoned animal has not been established but is implied by these data.

Effect of Low Temperature (5° C) on Protection of Mice Against Endotoxin

As the data of Table IX show, mice exposed to 5° C and injected with about an LD_{25} of S. marcescens LPS fail to show 100% survival when either nicotinamide, DPN, or cortisone is given at the same time. This is contrary to what was reported in Tables I and II with data from mice maintained at 25° C. By increasing the quantity of LPS to slightly more than an LD_{80}, nicotinamide and cortisone were able to protect the mice but DPN was still ineffective. This finding with cortisone in which protection occurs with a large dose but not a small dose of endotoxin confirms an earlier observation reported by Previte and Berry (1962; in press). These authors also observed that death from endotoxin in mice at 5° C occurs sooner and the animals fail to show the conjunctivitis, the ruffled fur, and the malaise seen in mice at 25° C. The vascular changes typically associated with endointoxication are believed not to occur in the cold-exposed mice.

TABLE VIII

Effect of heat-killed S. typhimurium, cortisone and nicotinamide singly and in combination on total oxidized pyridine nucleotides in liver of mice. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses. The P-values were calculated by the rank order test of White (1952).

17 Hours After Treatment with	Total Oxidized Pyridine Nucleotides mg/g Liver	Significant Difference (Probability) versus	
		Control Group	Endotoxin Group
Control	0.75 ± 0.04 (15)	--	.001
Endotoxin, 1.5 mg S. typhimurium	0.55 ± 0.06 (15)	.001	--
Cortisone acetate 5 mg	0.84 ± 0.08 (10)	.001	.001
Nicotinamide, 10 mg	1.95 ± 0.76 (9)	.001	.001
Cortisone + endotoxin	0.68 ± 0.09 (10)	N.S.	.01
Nicotinamide + endotoxin	0.78 ± 0.12 (10)	N.S.	.001

TABLE IX

Effect of exposure of mice to 5° C on ability to protect against the lethal effect of S. marcescens lipopolysaccharide. All injections were given intraperitoneally. The P-values were calculated by the rank order test of Wilcoxon (1949).

Substance Injected at Time of Endotoxin	Number of Mice Surviving/Number Injected with Quantity of S. marcescens LPS Shown	
	10 μg	30 μg
Control, saline 0.5 ml	22/30	5/30
Nicotinamide, 10 mg (82 μM)	24/30 N.S.	15/30 P = 0.01
DPN, 10 mg (17.1 μM)	26/30 N.S.	7/30 N.S.
Cortisone, 10 mg (18 μM)	25/30 N.S.	14/30 P = 0.02

Tryptophan Pyrrolase Activity in Mice at 5° C

After four hours of exposure to 5° C, a time at which the body temperature of mice is one to two degrees lower than normal (Previte and Berry, in press), the tryptophan pyrrolase activity of liver is significantly less than that of mice maintained at 25° C. This is seen in the first line of Table X. By 17 hours, however, the enzyme is again normal, as is body temperature (second line, Table X). The injection of the LD_{50} dose (at 25° C) of heat-killed S. typhimurium into mice at 5° C fails to alter after four hours the activity of tryptophan pyrrolase, while the same amount of crude endotoxin in mice at 25° C raises significantly the enzyme level (line 3, Table X). This quantity of endotoxin is about 200 times the LD_{50} for mice at 5° C and hence is not directly comparable to the results found at room temperature. By reducing the amount of endotoxin to less than an LD_{100}, there are increases in tryptophan pyrrolase levels in livers of mice in the cold (this can be seen in the last three lines of Table X) and the increases occur following injection of either heat-killed S. typhimurium or S. marcescens LPS.

TABLE X

Tryptophan pyrrolase activity of liver from mice at 5° C injected with different doses of endotoxin. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental Treatment	Tryptophan Pyrrolase Activity of Liver (μM Kynurenine/g Liver/Hour) for Mice Exposed to	
	5° C	25° C
After four hours exposure	9.1 ± 1.7 (8)	14.9 ± 1.5 (16)
After 17 hours exposure	13.8 ± 3.2 (9)	14.9 ± 1.5 (16)
Four hours after 1.5 mg heat-killed S. typhimurium	11.3 ± 3.2 (14)	21.3 ± 3.7 (8)
Four hours after 60 μg heat-killed S. typhimurium	12.6 ± 2.6 (8)	
Four hours after 6 μg heat-killed S. typhimurium	17.2 ± 2.6 (8)	
Four hours after 50 μg S. marcescens LPS	14.7 ± 3.5 (8)	

SECTION 4. DISCUSSION

The changes found to occur in liver tryptophan pyrrolase activity in mice given toxic amounts of endotoxin suggest that this enzyme and the metabolic pathway in which it participates are involved in endointoxication. When mice are protected against the lethal effects of endotoxin by cortisone, tryptophan pyrrolase activity remains normal. Tryptophan, on the other hand, augments the level of the enzyme in unpoisoned animals but fails to protect against lethality and to maintain the normal activity of the enzyme in the presence of endotoxin. Moreover, the ability of nicotinamide and DPN each to protect against endotoxin is additional evidence for a significant role of tryptophan pyrrolase in the survival of these animals. The extent to which enzymic changes reflect adrenocortical function remains to be established, but there seems to be little question that they are related. The rise in tryptophan pyrrolase activity about four hours after an injection of endotoxin (Figure 2) fails to occur in the adrenalectomized mouse. In addition, the minimum level of enzyme activity seen about 17 hours after the endotoxin is the same as that observed in the adrenalectomized animal. It is not unreasonable to postulate that the high susceptibility of the adrenal deprived animal to endotoxin is related to this metabolic deficiency.

The cold exposed animal presents an interesting exception to the behavior of mice maintained at room temperature. They are not protected against endotoxin by nicotinamide. In addition, these animals die from an LD_{50} dose sooner than mice at 25° C. It is known that the poisoned animals become severely hypothermic within a few hours (Previte and Berry, in press) and their death may be a direct result of this change. A number of factors may contribute to this loss of temperature, but one factor of significance is the suppression of gastric emptying (and hence cessation of food and water intake) which is demonstrable five minutes after as little as one-thousandth of an LD_{50} of endotoxin. Since mice in the cold eat nearly five times as much food as normal animals, it is apparent that inanition becomes an important consideration in survival.

The potentiating effect of a delayed injection of tryptophan (Table III) merits comment. Tryptophan is known to be degraded not only via its pyrrolase but it is also converted into 5-hydroxytryptamine (serotonin). Since this substance may sensitize an animal to endotoxin (Meeker and Davis, 1961), then the transformation of a greater proportion of tryptophan to serotonin in poisoned mice than in controls would account for the observed effect. Experimental verification is possible and experiments are now underway.

Recent publications by Martini (1959), Weissmann and Thomas (1962) and Janoff et al (1962) have strongly implicated lysosomes as a primary target of endotoxin action. Increased release of hydrolases from the so-called large granule fraction (LGF) of liver was demonstrated only five minutes after an intraperitoneal injection of bacterial lipopolysaccharide. Endotoxin tolerant animals, cortisone pretreated animals and rats made tolerant to traumatic shock all possess lysosomes from which less cathepsin and β-glucuronidase are released following incubation or ultraviolet irradiation than in preparations derived from control animals. Apparently the development of stability of lysosomes is related to adrenocortical integrity. Whether or not these observations are in any way related to our findings remains to be determined.

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